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Fernanda M. Ferreira [a] [b], Lia T. Dinis [c], Pedro Azedo [b], Cristina I.C. Galhano [a] [b], Anabela Simões [d], Susana M. Cardoso [a], M. Rosário [a], M. Domingues [e], Olívia R. Pereira [a] [f], Carlos M. Palmeira [d] & Francisco P. Peixoto [g]

[a] Center for Study of Natural Resources, Environment and Society (CERNAS), Coimbra, Portugal

[b] Department of Environment, Agricultural College of Coimbra (ESAC), Coimbra, Portugal

[c] Department of Biological and Environmental Engineering (DEBA), Centre for Investigation and Agro Environmental and Biological Technologies (CITAB), University of Trás-os-Montes & Alto Douro, Vila Real, Portugal

[d] Department of Life Sciences, Center for Neurosciences and Cell Biology of Coimbra, University of Coimbra, Portugal

[e] Department of Chemistry & QOPNA, University of Aveiro, Aveiro, Portugal

[f] Department of Diagnostic and Therapeutic Technologies, School of Health Sciences, Polytechnic Institute of Bragança, Bragança, Portugal

[g] Chemistry Department & CECAV, University of Trás-os-Montes & Alto Douro, Vila Real, Portugal

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Antioxidant capacity and toxicological evaluation of *Pterospartum tridentatum* flower extracts

Capacidad antioxidante y evaluación toxicológica de extracto de flores de *Pterospartum tridentatum*

Fernanda M. Ferreira[a,b]*[,†], Lia T. Dinis[c], Pedro Azedo[b], Cristina I.C. Galhano[a,b], Anabela Simões[d], Susana M. Cardoso[a], M. Rosário M. Domingues[e], Olívia R. Pereira[a,f], Carlos M. Palmeira[d] and Francisco P. Peixoto[g]

[a]*Center for Study of Natural Resources, Environment and Society (CERNAS), Coimbra, Portugal;* [b]*Department of Environment, Agricultural College of Coimbra (ESAC), Coimbra, Portugal;* [c]*Department of Biological and Environmental Engineering (DEBA), Centre for Investigation and Agro Environmental and Biological Technologies (CITAB), University of Trás-os-Montes & Alto Douro, Vila Real, Portugal;* [d]*Department of Life Sciences, Center for Neurosciences and Cell Biology of Coimbra, University of Coimbra, Portugal;* [e]*Department of Chemistry & QOPNA, University of Aveiro, Aveiro, Portugal;* [f]*Department of Diagnostic and Therapeutic Technologies, School of Health Sciences, Polytechnic Institute of Bragança, Bragança, Portugal;* [g]*Chemistry Department & CECAV, University of Trás-os-Montes & Alto Douro, Vila Real, Portugal*

Pterospartum tridentatum Willk. (prickled broom) is an autochthonous plant, common in Portuguese territory. The yellow flowers are used in traditional medicine, as a potential cure for all body illnesses, mainly to treat throat irritations or for diabetes, hypertension and hypercholesterolemia therapy. Despite its traditional use, no toxicological assessment has been performed to our knowledge. A high antioxidant activity of *P. tridentatum* flower water extract was found, in good agreement with its electrospray ionisation-mass spectroscopy (ESI-MS) spectrum which revealed the presence of several flavonoids, such as luteolin-*O*-(*O*-acetyl)-glucuronide, luteolin-*O*-glucuronide or isorhamnetin-*O*-hexoside. Mitocondrial respiratory rates (state 4, state 3 and FCCP-stimulated respiration) and respiratory indexes (respiratory control and P/O ratios) showed no consistent decrease of respiratory and phosphorylative efficiencies for the concentrations tested (up to 500 μg.mL^{-1}). Cytotoxicity evaluation, using MTT assay, was in agreement with the previous results. In conclusion, for the concentration range commonly used, *P. tridentatum* flower usage can be regarded as harmless and credible.

Keywords: antioxidant capacity; cytotoxicity; oxidative phosphorylation; phytotherapy; *Pterospartum tridentatum* (L.); Willk.; toxicologic evaluation

Pterospartum tridentatum Willk. es una planta autóctona común en el territorio portugués. Las flores amarillas se usan en medicina tradicional como cura potencial para todas las enfermedades, especialmente para tratamientos de irritación de garganta o para tratamientos contra diabetes, hipertensión o hipercolesterolemia. A pesar de su uso tradicional, ninguna evaluación toxicológica se ha llevado a cabo. Se encontró una alta actividad antioxidante de extracto acuoso de flor de *P. Tridentatum*, concordando con su espectro ESI-MS, el cual reveló la presencia de varios flavonoides, como luteolina *O*–(*O*–acetil)–glucurónido, luteolina–*O*–glucurónido o isorramntina–*O*–hexósido. Las frecuencias respiratorias mitocondriales (estado 4, estado 3 y respiración estimulada por FCCP) mostraron un descenso no consistente de eficiencia respiratoria y fosforilativa para las concentraciones analizadas (hasta 500 μg.mL^{-1}). La evaluación de citotoxicidad, usando ensayo MTT, coincidió con los resultados previos. En conclusión, para las concentraciones comúnmente empleadas, el uso de las flores de *P. tridentatum* se puede considerar inocuo y verosímil.

Palabras claves: capacidad antioxidante; citotoxicidad; fosforilación oxidativa; fitoterapia; *Pterospartum tridentatum* (L.) Willk; evaluación toxicológica

Abbreviations: ESI, electrospray ionization; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HepG2, a human hepatoma cell line; MS, mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; P/O ratio, ratio of ADP molecules phosphorylated to atoms of oxygen consumed; RCR, respiratory control ratio (state 3 respiratory rate/state 4 respiratory rate)

Introduction

Pterospartum tridentatum (L.) Willk., a *Fabacea* known as "prickled broom", (previously named *Chamaespartum tridentatum*) is an autochthonous plant of the Northwest part of Iberian Peninsula and Morocco. This plant is commonly found in Portuguese mountains and is locally known as "carqueja" or "carqueija".

P. tridentatum grows in acidic soils, in brushwoods and thickets. It is a shrub, with characteristic yellow flowers with a typical odor, that are traditionally harvested during Spring time, from March to June. Leaves and stems are normally used in cooking, to flavor rice, roast meat or hunting animals (Grosso et al., 2007). Dried stems are also used as firewood in traditional ovens because they are highly combustible, giving

an enjoyable aroma to bread (Grosso et al., 2007). Further, recent studies suggest that stems can also be used as a bioethanol source, in alternative to petroleum-derived fuels (S. Ferreira, Gil, Queiroz, Duarte, & Domingues, 2010). The yellow flowers are used in traditional medicine, sometimes in herbal mixtures, for throat irritation treatment, diabetes therapy or for controlling hypertension and hypercholesterolemia (Camejo-Rodrigues, Ascensão, Bonet, & Vallès, 2003; Castro, 1998; Grosso et al., 2007; Neves, Matos, Moutinho, Queiroz, & Gomes, 2009). As a matter of fact, the *P. tridentatum* flowers tea is widely used in Portuguese territory as a panacea, being regarded as a potential cure for all illnesses of the body. Additionally, *P. tridentatum* flowers infusion is also used in cooking to flavor rice (Grosso et al., 2007).

Despite a previous characterization of *P. tridentatum* flowers decoction (Vitor et al., 2004), it must be highlighted that, due to the existence of aromatic plant chemotypes, secondary metabolites present in plant extracts may vary (Díaz-Reinoso, Moure, Domínguez, & Parajó, 2007). Mass spectrometry (MS) with electrospray ionization (ESI) is a high sensitive technique (Griffiths, Jonsson, Liu, Rai, & Wang, 2001) frequently applied for the identification of mixture analytes. Considering that the ESI ionization is extremely soft, no chemical reaction is expected to occur during the ionization process. Recently, electrospray ionization-mass spectrometry with direct infusion of samples has appeared as a new alternative method for a fast and reliable fingerprint characterization of multicomponent polar natural product extracts, in particular those enriched in phenolic compounds (Cardoso, Falcão, Peres, & Domingues, 2011; Roesler, Catharino, Malta, Eberlin, & Pastore, 2007). Moreover, the coupling with tandem mass spectrometry (MS/MS) of the diagnostic ions, allows providing detailed structural information on the sample components.

Medicinal plants seem to be a useful alternative or complement to the synthetic drugs used in most chronic diseases therapy (Hassan, Imran, Irfan, & Loon, 2009). Several synthetic drugs, such as metformin, guanidine or aspirin, are plant based active compounds previously extracted from medicinal plants (Mueller & Jungbauer, 2009; Petlevski, Hadzija, Slijepcevic, & Juretic, 2001). Only recently, investigation on toxicological effects of some medicinal plants has started (Aguilar-Santamaría et al., 2007; Déciga-Campos et al., 2007); however, for most plants, their potential toxicological effects remain poorly characterized (Jordan, Cunningham, & Marles, 2010).

Liver is the major organ responsible for detoxification processes and mitochondria constitute the major energy-producing organelles of the cell. Any interference with mitochondrial bioenergetics is known to be a part of cell injury process by a multiplicity of mechanisms and assorted agents (Wallace, 2008; Wallace, Eells, Madeira, Cortopassi, & Jones, 1997). Mitochondria has proven to be an excellent model to evaluate the many xenobiotics' effects on cell toxicity and the obtained data are usually in good agreement with cytotoxicity parameters reported in cell cultures and whole organisms (Haubenstricker, Holodnick, Mancy, & Brabec, 1990; Knobeloch, Blondin, Read, & Harkin, 1990). Based on these facts, the purpose of the present study was the assessment of potential toxicological effects of *P. tridentatum* flowers water extracts. To achieve this, mitochondrial parameters (state 3, state 4, uncoupled respiration, respiratory control ratio (RCR), and P/O ratio) and mitochondrial inner membrane integrity were evaluated in the presence of different amounts of plant extract. This study was further complemented with cytotoxicological evaluation of *P. tridentatum* flowers water extract in HepG2 cells, a human hepatocellular liver carcinoma cell line.

Materials and methods

Materials

The human hepatoma cell line (HepG2) was obtained from American Type Culture Collection (ATCC, Rockville, MD). All chemicals used were of analytical grade and obtained from standard commercial sources. Inhibitors and drugs were dissolved in water or ethanol. In control experiments, solvents were added to isolated mitochondria at concentrations not exceeding 0.2%.

Plant material

The plants used in this study were directly obtained from "Ervital – Plantas Aromáticas e Medicinais, Lda" (Castro Daire, Portugal), and collected in the Castro Daire region (Portugal). They were air-dried in the dark. Vouchers of specimens (*Rosmarinus officinalis* leaves n° HVRG000052; *Pterospartum tridentatum* flowers n°HVRG000051) were deposited in the University of Trás-os-Montes and Alto Douro Herbarium (Vila Real, Portugal).

Water plant extracts preparation

The leaves or flowers of the plants, used in the study, were washed in deionized water and minced to small pieces. Water extracts were prepared by mixing 50 g of the dried material in 1000 mL of ultrapure water; the mixture was homogenized in a blender for 10 min and then stirred for 2 h at room temperature. The homogenates were filtered twice through a gauze tissue and centrifuged at 7000 g for 5 min at 4°C. Pellets were discarded. Part of the supernatants was kept at -20°C and the remainder was lyophilized and kept at -20°C.

β-carotene bleaching test

Antioxidant activity was determined using the β-carotene bleaching test (Ismail, Marjan, & Foong, 2004), with some modifications. Briefly, 1 mL of β-carotene solution (0.2 g.L^{-1} in chloroform) was added to 20 μL linoleic acid and 200 μL of 100% Tween-80. This mixture was kept in the dark till evaporation, at 40°C for 10 min, in a rotary evaporator, to remove chloroform. Then, it was immediately diluted with 100 mL of oxygenated distilled water which was slowly added to the mixture and vigorously stirred to form an emulsion. Five milliliters of the emulsion was transferred into different test tubes containing 400 μL of samples. Distilled water was used as control. The tubes were then gently shaken and placed in a water bath, at 40°C for 60 min. The absorbance of the samples, standard and control was measured at 470 nm using a Perkin-Elmer Lambda 40 UV/VIS spectrophotometer against a blank, an emulsion without β-carotene. Measurement was carried out at initial time ($t = 0$), at 30 and 60 min. Three different extracts from each plant were assayed in triplicate and averaged. The antioxidant activity (AA) was

measured in terms of β-carotene successful bleaching using the following equation: AA $= \{1-[(A_0-A_t)/(A°_0 - A°_t)]\} \times 100$, where A_0 and $A°_0$ are the absorbance values measured at the initial incubation time for samples/standard and control, respectively, while A_t and $A°_t$ are the absorbance values measure in the samples/standard and control, respectively, at $t = 30$ and 60 min.

Assay of ABTS radical scavenging activity

ABTS radical-scavenging activity of the hydrophilic fractions was determined by a procedure previously reported (Miller & Rice-Evans, 1997), with slight modifications. The ABTS$^{·+}$ solution was prepared by mixing 7 mM of ABTS salt with 3 mM of potassium persulfate in 25 mL of distilled water. The solution was held at room temperature, in the dark, for 16 h before use. The ABTS$^{·+}$ solution was diluted with 95% ethanol (approximately 600 μL ABTS to 40 mL 95% ethanol), in order to obtain an absorbance between 0.8 and 0.9 at 734 nm. Fresh ABTS$^{·+}$ solution was prepared for each analysis. Antioxidant or standard solutions, 50 μL (corresponding to 2500 mg.L^{-1}), were mixed with 1 mL of diluted ABTS$^{·+}$ solution and incubated at 30°C. The absorbance at 734 nm was read after 6 min. Ethanol (95%) was used as a blank. Trolox, at 0 to 500 μM, was used as standard and the free-radical scavenging activity was expressed as Trolox equivalents (TE). Triplicates of three different plant extracts prepared in similar conditions were evaluated.

Determination of antioxidant contents

Total phenolic compounds in the water extracts (or in lyophilized extracts) were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (Singleton & Rossi, 1965), with some modifications, accordingly to Barreira and colleges (Barreira, Ferreira, Oliveira, & Pereira, 2008), for lyophilized extracts: 0.5 g of extracts were mixed with ultrapure water or ethanol 50% and shaken until a clear solution was obtained. This step was repeated three times to increase the extraction yield. All the samples were redissolved in the same solvent at a concentration of 20 g.L^{-1} and analyzed for their phenolics compounds and flavonoids.

Basically, 0.2 mL of sample was mixed with 1 mL of Folin–Ciocalteu's phenol reagent. After 30 sec, 0.8 mL of 7.5% sodium carbonate solution was added. The reaction was kept in the dark for 30 min, then, absorbance was read at 765 nm, using a UV-VIS spectrophotometer. Gallic acid (0–20 μg) was used as a standard. Results were expressed as μg of gallic acid equivalents (GAE) of extract (mL^{-1} or g^{-1}).

Flavonoid contents in the extracts were determined by a colorimetric method described by Jia et al. (Jia, Tang, & Wu, 1999), with some modifications (Barreira et al., 2008). *P. tridentatum* and *R. officinalis* extracts (250 μL of lyophilized extract reconstituted in ultrapure water or ethanol 50%) were mixed with 1.25 mL of distilled water and 75 μL of a 5% NaNO$_2$ solution. After 5 min, 150 μL of 10% AlCl$_3$ · H$_2$O solution was added. After 6 min, 500 μL of 1 M NaOH and 275 μL of distilled water were added to prepare the mixture. The solution was well stirred and the absorbance was read at 510 nm. (+)-Quercetin was used as a standard to obtain the standard curve (0–2.5 mM; $y = 0.0099x + 0.0094$;

$R^2 = 0.9952$) and the results were expressed as mg of (+)-quercetin equivalents (QE) of extract (g^{-1}).

Mass spectrometry analysis of the Pterospartum tridentatum flower water extract

MS analysis of *P. tridentatum* flower extract was determined according to a method previously described (Cardoso et al., 2011). The freeze-dried aqueous extract was dissolved in methanol and directly injected into the ESI source by means of a syringe pump, at flow rate of 8 μL.min^{-1}. ESI-MS analyses were performed in the negative ion mode within the m/z range 50–1000, using a Linear Ion trap LXQ instrument (ThermoFinnigan, San Jose, CA, USA) equipped with Xcalibur® software (ThermoFinnigan, San Jose, CA, USA). Typical ESI conditions were: nitrogen sheath gas 30 psi, spray voltage 4.7 kV, capillary temperature 275°C, capillary voltage -37.0 V and tube lens voltage -81.89 V. CID-MS/MS and MS^n experiments were performed on mass-selected precursor ions using standard isolation and excitation configuration. Full scan data acquisition was performed from m/z 100 to m/z 1000 in MS scan mode. The collision energy used was between 10 and 40 (arbitrary units).

Animals

Wistar rats (200–300 g) were fasted overnight before being killed by cervical displacement. The isolation of mitochondria was performed by conventional methods (Gazotti, Malmstron, & Crompton, 1979), with minor modifications. The homogenization medium contained 0.25 M sucrose, 5 mM Hepes (pH 7.4), 0.2 mM EGTA and 0.1% fatty acid-free bovine serum albumin (BSA). EGTA and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. The final concentration of mitochondrial protein was determined by the biuret method (Gornall, Bardawill, & David, 1949), using BSA as standard. The experiments were carried out in accordance with the National Requirements for Vertebrate Animal Research and the EU guidelines (2010/63/EU).

Mitochondrial respiration

Oxygen consumption of isolated mitochondria was determined polarographically at 30°C with a Clark oxygen electrode, connected to a suitable recorder in a closed chamber with magnetic stirring (Estabrook, 1967). Plant water extracts were added in aliquots (0–10 μL) to 1 mL of the standard respiratory medium (30°C) with mitochondria (1 mg protein), supplemented with 2 iM rotenone and allowed to incubate for 5 min before the addition of 10 mM succinate. State 3 was elicited by adding adenosine 5'-diphosphate (ADP; 100 nmol) and state 4 respiration was achieved after phosphorylation of 100 nmol ADP (Chance & Williams, 1956). For uncoupled respiration, 1 μM FCCP was added after a phosphorylative cycle. Controls were made as above with minor changes: first mitochondria were exposed to a medium supplemented with substrate and 5 min later, 500 mg.L^{-1} of plant extract was added. The purpose of this control was to verify whether the mitochondria pre-incubated with plant water extract, prior to substrate addition, promoted an irreversible damage on the respiratory

complexes, with maximal respiratory rates limitation. In order to validate respiratory activity assays, 1 mM KCN was added and the slope due to O_2 diffusion was subtracted.

Mitochondrial swelling

Mitochondrial osmotic volume changes were measured by the apparent absorbance changes at 520 nm with a suitable spectrophotometer-recorder set up. Mitochondrial swelling methods were used to detect H^+ mitochondrial inner membrane permeabilization (Diogo et al., 2009). Reactions were carried out at 30°C, with 1 mg mitochondrial protein suspended in 3.0 mL of the required media, as described below.

Mitochondrial inner membrane permeabilization to H^+, due to *P. tridentatum* extract, was detected in potassium acetate medium (135 mM K-acetate, 5 mM Hepes (pH 7.1), 0.1 mM EGTA, and 0.2 mM EDTA) supplemented with 2 μM rotenone. All assays were performed in the presence of 1 μg.mL^{-1} valinomycin to permeabilize to K^+ ions. A control assay was performed in the presence of 1 μM FCCP for total permeabilization to H^+ ions.

Cell culture

HepG2 cells were cultured in Minimum Essential Medium (MEM) Eagle (with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g.L^{-1} sodium bicarbonate, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate), containing 10% fetal bovine serum. Cells were maintained in a humidified CO_2 incubator at 37°C, passaged and harvested for experiments by detachment with 0.05% trypsin and 0.5 mM EDTA in phosphate-buffered saline (PBS); 80–90% confluent cells were used for *P. tridentatum* treatment.

Cell cytotoxicity assays

Cytotoxicological effects of plant extracts was evaluated through MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) assay, which has been widely used as a colorimetric approach based on the activity of mitochondrial dehydrogenase enzymes in cells. HepG2 cells were inoculated in 96-well plate and at 50% confluency they were treated with 125 and 375 mg.L^{-1} of *P. tridentatum* water extracts, as indicated in figure legends and subjected to MTT assay after 24 and 48 h (Mosmann, 1983). The resultant product was quantified by spectrophotometry, using a plate reader at 570 nm.

Statistics

The results are presented as mean $\pm$ SD (standard deviation) or SEM (standard error of mean) of the number of experiments shown on tables and figures legends. Statistical significance was determined using Student *t*-test and for multiple comparisons statistical significance was determined using one-way ANOVA, with Tukey post-test. $p < 0.05$ was considered significant.

Results

The antioxidant activity of crude *P. tridentatum* flower extracts were determined and compared to *R. officinalis* leaf extracts, prepared in similar conditions. *R. officinalis* water extract was used as a positive control in antioxidant assays, as it is regarded as a common aromatic and medicinal plant with high antioxidant activity.

Both extracts presented similar antioxidant activity, as shown by ABTS radical scavenging activity and β-carotene bleaching test (Table 1). However, β-carotene bleaching evaluated 60 min later, showed that *P. tridentatum* presents a significantly higher antioxidant activity than *R. officinalis* (170 and 112%, respectively). This result is in good agreement with the amount of total phenolic compounds present in *P. tridentatum*, evaluated either from water or lyophilized extracts (Figure 1A and B), compared to *R. officinalis*. In fact, in *P. tridentatum* lyofilized extracts phenols content (expressed as gallic acid equivalents) is significantly high, extracted either with water or ethanol 50%, in order to solubilize less hydrophilic components. Furthermore, these extracts present a higher amount of flavonoids (Figure 1C), when compared to *R. officinalis*.

The ESI-MS spectrum of the water extract of *P. tridentatum* flowers (data not shown) revealed a mixture of compounds which enclosed the cyclic polyolquinic acid (MW 192 Da), the cyclopentyl fatty acid derivatives 12-hydroxyjasmonate sulfate and 5′-hydroxyjasmonic acid 5′-O-glucoside (MW 306 Da and 388 Da, respectively) and various phenolic compounds. Regarding the latter components, main $[M-H]^-$ ions were observed at m/z 359, 461, 477, 503, and 641. The ESI-MS^n data of these phenolic components, as well as their tentative structures are resumed in Table 2. Attending that the mentioned phenolic compounds were detected as abundant ions in ESI-MS spectrum, data suggests that *P. tridentatum* flowers are enriched in phenolics of distinct classes, including phenolic acids, flavones, flavonols, and isoflavones.

In more detail, the $[M-H]^-$ ion at m/z 359 corresponded to rosmarinic acid, as its fragmentation pattern was similar to that of the standard compound (major product ions in the MS^2 spectrum were observed at m/z 161, 197, and 223). Alternatively, the $[M-H]^-$ ion at m/z 461 was assigned to 5,5′-dihydroxy-3′-methoxy-isoflavone-7-O-ß-glucoside, as the main product ions of that ionic specie corresponded to those reported by Paulo et al. (2008). Moreover, ions at m/z 477

Table 1. Antioxidant activities of *P. tridentatum* flower extract.

Tabla 1. Actividad antioxidante de extracto de flor de *P. tridentatum*.

Plant extract	ABTS (TE, mmol.g^{-1})	β-Carotene bleaching test (% AA)	
		30 min of incubation	60 min of incubation
Pterospartum tridentatum	143.0 $\pm$ 0.8	101.8 $\pm$ 10.7	169.5 $\pm$ 17.2*,#
Rosmarinus officinalis	144.8 $\pm$ 0.4	112.3 $\pm$ 12.3	111.6 $\pm$ 12.6

Note: Results are presented as mean $\pm$ SD of triplicates of experiments performed with four plant water extracts prepared in similar conditions. Statistics: *$p < 0.05$ as compared to control; #$p < 0.05$ as compared to *R. officinalis*.

Nota: Los resultados son la media $\pm$ desviación estándar de experientos llevados a cabo con extracto acuoso de cuatro plantas preparados en condiciones similares. Estadísticas: *$p < 0,05$ comparado con control; #$p < 0,05$ comparado con *R. officinalis*.
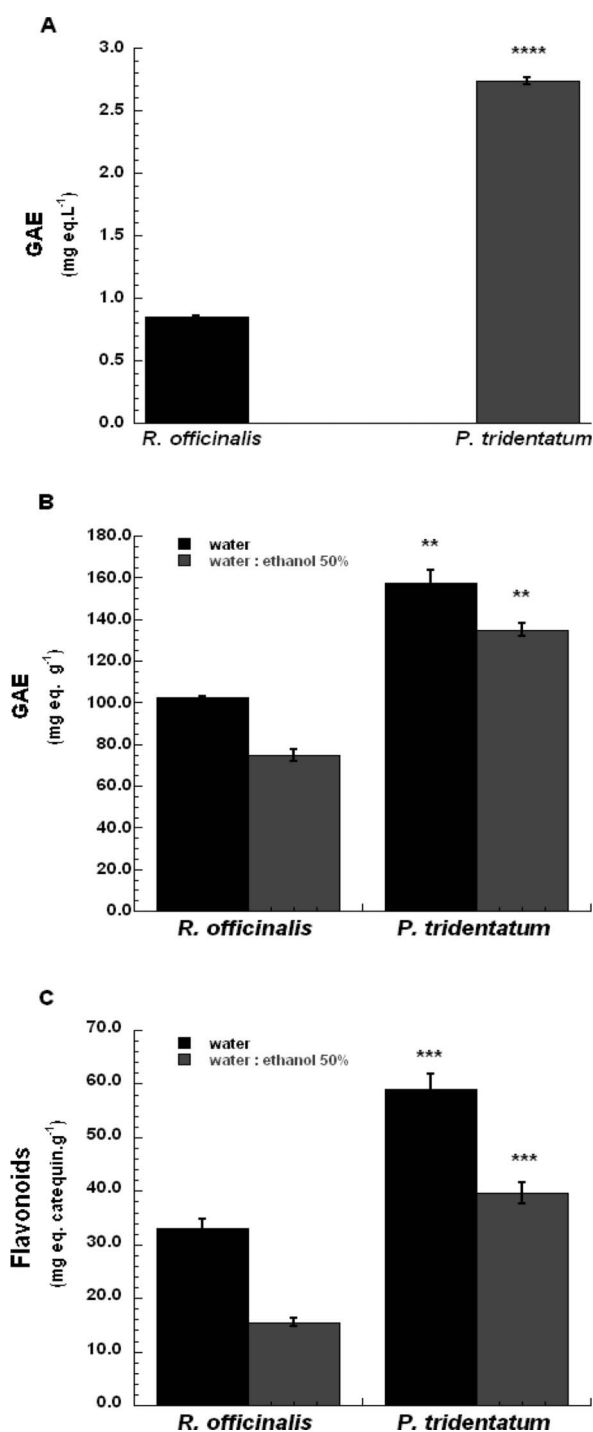
Figure 1. Phenols and flavonoids quantification in *P. tridentatum* and *R. officinalis* water extract. Phenolic content was determined from crude extract (A) and lyophilized extract (B). Flavonoids were determined from lyophilized extract (C). Lyophilized extracts were resuspended in water or in ethanol 50%. Results are presented as mean ± SD of triplicates. Statistics: **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, as compared to *R. officinalis* extract.

Figura 1. Cuantificación de fenoles y flavonoides en extracto acuoso de *P. tridentatum* y *R. officinalis*. El contenido de fenoles se determinó por extracto no purificado (A) y extracto liofilizado (B). Los flavonoides se determinaron por extracto liofilizados (C). Los extractos liofilizados se resuspendieron en agua o en etanol 50%. Los resultados se presentan como media ± desviación estándar de triplicados. Estadísticas: **$p < 0,01$; ***$p < 0,001$; ****$p < 0,0001$, comparado con extracto de *R. officinalis*.

and 641 were flavonols glucoside derivatives. Indeed, the MS/MS spectra of the ion at m/z 477 was coherent with an isorhamnetin glucoside. The presence of a hexose moiety in the molecule was confirmed by the product ion at m/z 315 (-162 Da), while the loss of 15 Da (methyl group) was observed at the MS^3 spectrum of the latter ion. Moreover, the fragmentation patterns of the ions at m/z 315 and 300 were consistent with that described for isorhamnetin (Gouveia & Castilho, 2010). On the other hand, the flavonol with a molecular weight of 642 Da should correspond to a pentahydroxyflavonol-di-*O*-glucoside, as the MS^2 spectrum of the molecular ion at m/z 641 showed the ion at m/z 479 (-162 Da) and a less abundant ion at m/z 317 (loss of two glycosyl units).

The flavone detected as a major molecular ion (m/z 503) in the water extract of *P. tridentatum* flowers was assigned to luteolin-acetyl glucuronide. Accordingly, the MS^2 spectrum of that ion showed product ions resultant from the loss of an acetyl moiety (m/z 461) or a neutral glucuronide unit (m/z 285) as well as the loss of 104 Da (ion at m/z 399), which corresponds to the cleavage of the glucuronide residue. Furthermore, the MS^3 of the ion at m/z 285 corresponded to that of the luteolin standard (major product ions in the MS^2 spectrum were observed at m/z 243, 241, 217, 199, 175, and 151).
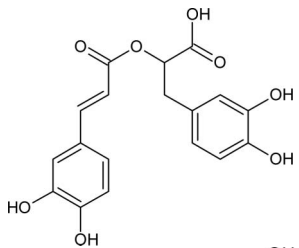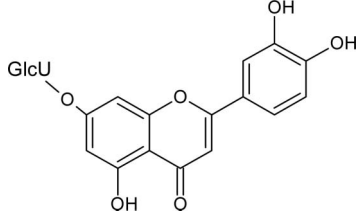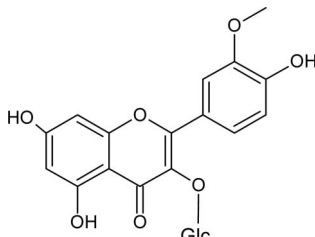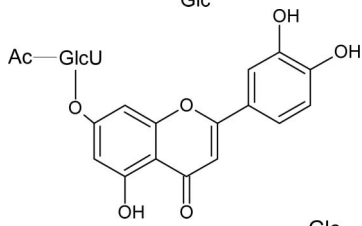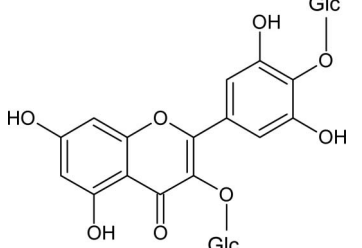
Crude *P. tridentatum* flower water extract were tested on isolated hepatic mitochondrial fractions with the purpose of determining their direct effects on mitochondrial bioenergetics (Table 3). Pre-incubation with plant water extract, did not affect mitochondrial phosphorylative oxidation (data not shown). State 4 respiration rate, according to Chance and Williams (1956), was determined upon the addition of succinate, a FAD-linked substrate (and in the presence of rotenone, a NADH:CoQ reductase or Complex I inhibitor, to ensure that NAD-linked substrates are not oxidized). State 3 respiration, in accordance to Chance and Williams (1956), was determined upon addition of exogenous ADP. At the end of the experiments, KCN (1 mM) was added to completely inhibit respiratory chain. If a slope different from zero was observed, due to O_2 diffusion, it was subtracted from the other respiratory rates.

Our results showed that *P. tridentatum* flower extract caused a slight decrease in state 4 and state 3 respiration rates. To evaluate the effect *P. tridentatum* flower extract over the respiratory chain (succinate-coenzyme Q reductase, coenzyme Q-cytochrome *c* reductase and cytochrome *c* oxidase or Complexes II, III, and IV), stimulated respiration in the presence of FCCP, a weak acid that behaves as an uncoupler – FCCPsr, was evaluated. We observed that FCCPsr, was not significantly affected by *P. tridentatum* extract for the tested concentrations (0–500 mg.L^{-1}; see Table 3), which seems to indicate an unspecified decrease of respiratory activity instead of a specific inhibition of respiratory complexes. The only exception was observed for the concentration of 50 mg.L^{-1}. Furthermore, no significant decrease in RCR or P/O ratio was observed (Figure 2), indicating no changes in phosphorylative efficiency.

The decrease in respiratory states 3 and 4 suggested a modification in membrane fluidity and permeability. Therefore, to evaluate the possible interactions between *P. tridentatum* flower extract hydrophobic compounds and the inner mitochondrial membrane, osmotic volume adjustments

Table 2. Resume of major ions in the ESI-MS spectrum of the water extract of *Pterospartum tridentatum* flowers corresponding to phenolic compounds, with the indication of the main product ions observed in their MS^n spectra and the proposed structures.

Tabla 2. Resumen de iones más importantes en el espectro ESI-MS de extracto acuoso de flores de *Pterospartum tridentatum* correspondiente a los componentes fenólicos, con la indicación de iones del principal producto observados en los espectros MS^n y estructuras propuestas.

[M-H]⁻	Main fragments, ESI-MS^n	Tentative structure	Compound
359	MS^2[359]: 315, 223, 197, 178, 161; MS^3[197]: 179, 73; MS^3[223]: 205, 179; MS^3[179]: 161, 135; MS^3[161]: 133		Rosmarinic acid
461	MS^2[461]: 285; MS^3[285]: 267, 257, 243, 241, 217, 199, 175, 151		Luteolin-*O*-glucuronide
477	MS^2[477]: 315, 300; MS^3[315]: 299, 300; MS^4[300]: 283, 272, 255, 243, 227, 216, 199		Isorhamnetin-*O*-hexoside
503	MS^2[503]: 461, 443, 399, 285; MS^3[285]: 267, 257, 243, 241, 217 , 199, 175, 151; MS^3[443]: 399, 381, 285; MS^3[399]: 355, 327, 285, 283; MS^4[283]: 255		Luteolin-*O*-(*O*-acetyl)-glucuronide
641	MS^2[641]: 479, 317/316/315, 301		Pentahydroxy-flavonol-di-*O*-glucoside

Note: GlcU, Glucuronide unit; Glc, Glucoside unit; Ac, Acetyl unit.

Nota: GlcU, Unidad Glucuronil; Glc, Unidad Glucosil; Ac, Unidad Acetil.

in the presence of plant extract (250 and 500 mg.L^{-1}) were evaluated. A slight decrease in absorbance was observed, revealing a slender increase in mitochondrial membrane permeability in the presence of the highest concentrations of plant extract used in this study (Figure 3).

In order to confirm that crude *P. tridentatum* flower extract presented no cytotoxicity in the range of concentrations studied, MTT assay was performed after an incubation of both 24 and 48 h, respectively with 125 and 375 mg.L^{-1} of water plant extract. This is a simple colorimetric assay to access cell viability, where in MTT, a yellow tetrazole, is

reduced by mitochondrial reductases to purple formazan in living cells (Mosmann, 1983). Our results showed no significant differences, when compared to control group, confirming no cytotoxicological effects for the studied range of concentrations (Figure 4).

Discussion

P. tridentatum is one of the most commonly used aromatic and medicinal species in Portugal, since ancient times. Despite being a plant usually found in the Northwest part

Table 3. Effects of *P. tridentatum* water extract on mitochondrial respiratory rates.

Tabla 3. Efectos de extracto acuoso de *P. tridentatum* en la frecuencia respiratoria mitocondrial.

P. tridentatum flower extract (mg.L^{-1})	Respiratory rate (nmol O$_2$.mg protein^{-1}.min^{-1})		
	State 4	State 3	FCCP-stimulated respiration
0	19.2 $\pm$ 1.0	73.1 $\pm$ 2.0	124.4 $\pm$ 4.4
25	11.5 $\pm$ 1.5	61.9 $\pm$ 5.2	128.0 $\pm$ 8.9
50	17.2 $\pm$ 2.3	60.6 $\pm$ 5.5*	115.2 $\pm$ 8.6
250	12.2 $\pm$ 0.8*	76.0 $\pm$ 4.3	142.7 $\pm$ 0.6
500	15.8 $\pm$ 1.4	60.9 $\pm$ 5.1*	132.0 $\pm$ 5.0

Note: Values of respiratory rates in state 3, state 4 and FCCP stimulated respiration (respectively V$_3$, V$_4$ and FCCP) are expressed as nmol O$_2$.mg protein^{-1}.min^{-1}. Results are presented as mean $\pm$ SEM of triplicates of experiments performed with four mitochondrial preparations. Statistics: *$p < 0.05$ as compared to control.

Nota: Los valores de la frecuencia respiratoria en estado 3, estado 4 y respiración estimulada por FCCP (V$_3$, V$_4$ y FCCP, respectivamente) se expresan como nmol O$_2$.mg proteina^{-1}.min^{-1}. Los resultados se presentan como media $\pm$ desviación estándar de la media de triplicados de experimentos llevados a cabo con cuatro preparaciones mitocondriales. Estadísticas: *$p < 0.05$ comparado con control.

of Iberian Peninsula and in Morocco, its therapeutic and flavoring use is almost restricted to Portuguese territory (Camejo-Rodrigues, et al., 2003; Neves et al., 2009; Pardo-de-Santayana et al., 2007). In fact, references to this plant from Spanish and Northwest African researchers are scarce (Bremner et al., 2009; Pardo-de-Santayana et al., 2007). Even so, only in the last decade the chemical characterization and evaluation of its therapeutic efficacy have been described (Bremner et al., 2009; Grosso et al., 2007; Luís, Domingues, Gil, & Duarte, 2009; Paulo et al., 2008; Vitor et al., 2004).

Previous studies point out that *P. tridentatum* flower and leaf decoctions present a high antioxidant activity and a large amount of phenols and flavonoid compounds (Luís et al., 2009; Vitor et al., 2004). Note that, from all the phenolic compounds determined in the *P. tridentatum* flower extract used in the present study, only 5,5'-dihydroxy-3'-methoxy-isoflavone-7-*O*-ß-glucoside has been previously detected in water extracts of *P. tridentatum* flowers (Vitor et al., 2004; Paulo et al., 2008). Moreover, from the remaining phenolic compounds described by those authors for water extracts of *P. tridentatum* flowers (myricetin-6-*C*-glucoside, rutin, iso-quercitrin, sissotrin, genistein, genistin, 7-methylorobol, and prunetin), only the latter three were detected in the present study. Their corresponding [M–H]$^-$ ions appeared as low intense signals in the MS spectrum (data not shown), thus suggesting that these compounds are minor components of the water extract of *P. tridentatum* flowers herein studied. Phenolic composition differences between the present studied extract and those previously reported would be easily understood if *P. tridentatum* chemotypes had been used. Indeed, as the sample flowers of the present work were collected at a distinct place and year from those of the previous works (Vitor et al., 2004; Paulo et al., 2008), the presence of chemotypes having a distinct phenolic composition must be considered.

The antioxidant properties of flavonoid compounds are related to their ability to chelate metal ions and scavenge singlet oxygen, superoxide, peroxyl, hydroxyl, and
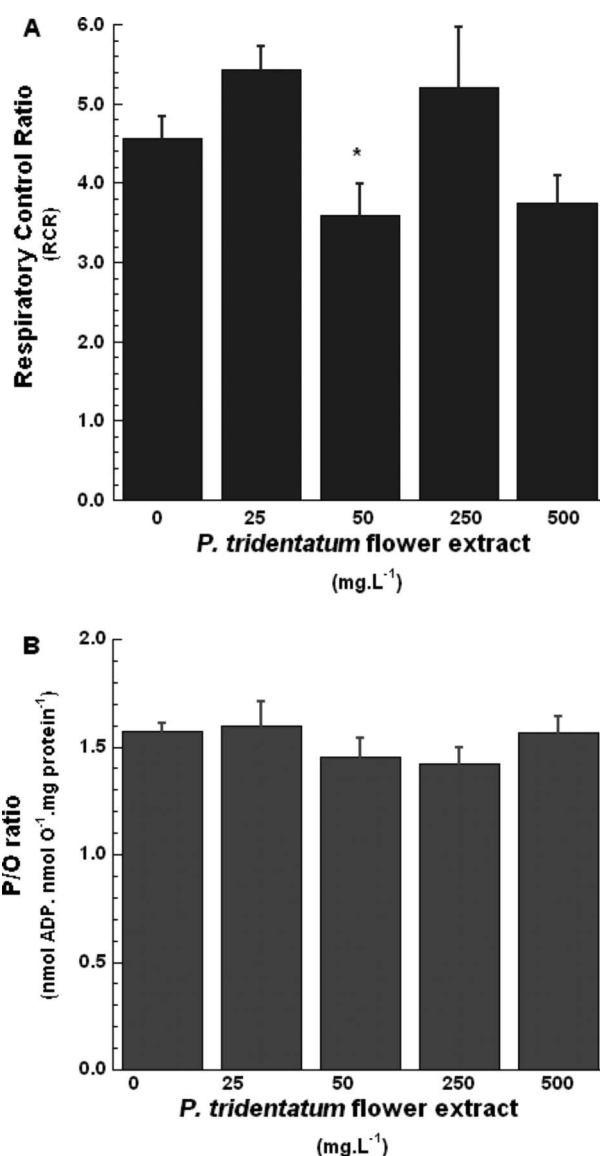

Figure 2. Effects of *Pterospartum tridentatum* flower treatment on liver mitochondria respiratory indexes: respiratory control ratio (RCR) (A) and P/O ratio (B). Mitochondria (1 mg protein) were incubated in 1 mL respiratory standard medium containing succinate (5 mM) and rotenone (1 μM), for 5 min at 30°C. State 3 respiration was initiated by the addition of 100 nmol ADP. Values are the means $\pm$ SEM of triplicates performed with four different mitochondrial preparations.

Figura 2. Efectos de tratamiento con flor de *Pterospartum tridentatum* en índices respiratorios de mitocondria de hígado: ratio control respiratorio (RCR) (A) y ratio P/O (B). Las mitocondrias (1 mg proteína) fueron incubadas en 1mL de medio estándar respiratorio con succinato (5 mM) y rotenona (1 μM), durante cinco minutos a 30°C. La respiración estado 3 se inició por la adición de 100 nmol ADP. Los valores son la media $\pm$ desviación estándar de triplicados llevados a cabo con cuatro preparaciones mitocondriales diferentes.

peroxynitrite radicals (Boveris & Puntarulo, 1998; Elingold et al., 2008; Rice-Evans, Miller, & Paganga, 1997, 1996), while phenolic compounds may prevent lipid peroxidation via hydrogen atom donation from the hydroxyl group(s) attached to the benzene ring (Sawa, Nakao, Akaike, Ono, & Maeda, 1999).
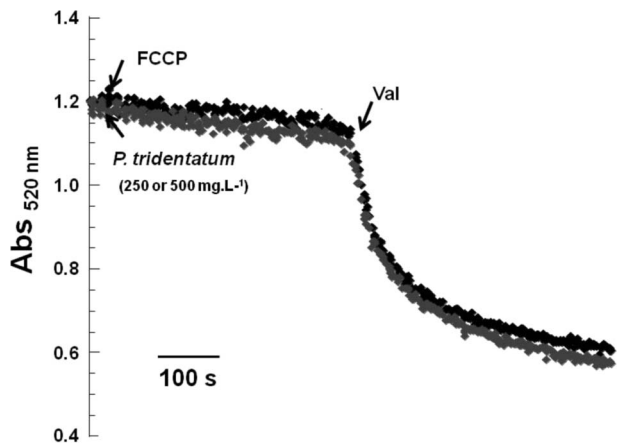
Figure 3. Effect of *P. tridentatum* flower water extract on the permeabilization to H^+ and K^+ by the inner membrane of rat liver mitochondria, evaluated by passive osmotic swelling of mitochondria suspended in potassium acetate. Freshly isolated liver mitochondria were incubated at 1 mg protein in 3 mL of appropriate medium, as described in the materials and methods section. Where indicated by arrows, 1 μM FCCP, *P. tridentatum* flower water extract (250 and 500 mg.L^{-1}) or 1 μg.mL^{-1} valinomycin (VAL) were added. The traces are typical recordings representative of three experiments obtained from different mitochondrial preparations.

Figura 3. Efecto de extracto acuoso de flor de *P. tridentatum* en la permeabilización de H^+ y K^+ por la membrana interna de mitocondria de hígado do rata, analizado por la turgencia producida por ósmosis pasiva de mitocondrias suspendidas en acetato de potasio. Mitocondrias de hígado recién aisladas se incubaron a 1 mg de proteína en 3 mL de medio apropiado, como se describe en la sección "Materials and Methods". Donde se indica con flechas, se añadió 1 μM de FCCP, extracto acuoso de flor de *P. tridentatum* o 1 μg.mL^{-1} de valinomicina (VAL). Las trazas son registros típicos representativos de tres experimentos obtenidos de diferentes preparaciones mitocondriales.
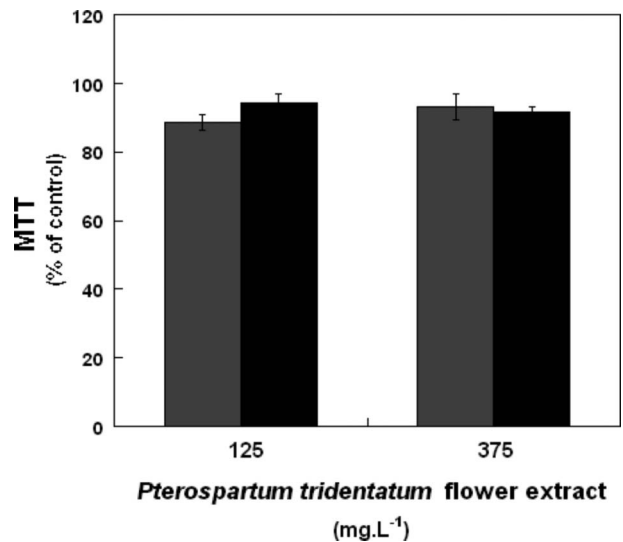

Figure 4. Effects of *P. tridentatum* flower water extract on cell viability. Cytotoxicity of *P. tridentatum* flower water extract was evaluated through MTT assay, which has been widely used as a colorimetric approach based on the activity of mitochondrial dehydrogenase enzymes in cells. MTT test was performed after an incubation of both 24 h (grey) or 48 h (black) with 125 and 375 mg.L^{-1} of water plant extract. Data represent the mean $\pm$ SD of measurements of three separate experiments.

Figura 4. Efectos de extracto acuoso de flor de *P. tridentatum* en viabilidad de célula. La citotoxicidad de extracto acuoso de flor de *P. Tridentatum* se evaluó a través de ensayo MTT, que se ha usado como propuesta colorimétrica basada en la actividad de enzima deshidrogenasa mitocondrial en células. El examen MTT se llevó a cabo tras una incubación de 24 horas (gris) o 48 horas (negro) con 125 y 375 mg.L^{-1} de extracto acuoso de planta. Los datos representan la media $\pm$ desviación estándar de tres experimentos diferentes.

In the present study, the antioxidant capacity of crude *P. tridentatum* flower water extract was evaluated and compared with *R. officinalis* leaf extract antioxidant activity, as this plant is one of the most common aromatic and medicinal plants with high antioxidant properties (Cheung, Wyman, & Halcon, 2007). The antioxidant capacity was evaluated "in vitro" using simple and suitable tests for vegetable samples (Sun, Powers, & Tang, 2007). ABTS assay was used to quantify the amount of hydrogen peroxide quenched in samples. Further, β-carotene bleaching is a free radical-mediated phenomenon resulting from the hydroperoxides formed from linoleic acid and was used to evaluate if radical scavenging activity are representative of plant extracts oxidation retard potential (Wang, Zhang, Duan, & Li, 2009). Our results indicate that, for a short period of time (up to 30 min), both extracts presented similar antioxidant capacities. However, for a longer period (60 min), *P. tridentatum* flower extract showed a significantly higher antioxidant activity using β-carotene bleaching assay. These results present good correlation with phenols and flavonoids contents from both extracts and with the previous reports (Luís et al., 2009; Vitor et al., 2004). As flavonoids and their metabolites have one-electron reduction potentials lower than those of highly oxidizing reactive oxygen species (ROS) (Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994), these compounds possess high ability to reduce these species.

Previous studies suggested that flavonoids present in *P. tridentatum* decoctions exhibited high antioxidant capacity and protected endothelial cells against oxidative injury and, thus, could prevent or reduce the development of diabetic vascular complications (Vitor et al., 2004). *P. tridentatum* flower decoction's importance as anti-inflammatory agent also seems to be related with the high amount of flavonoid compounds and to their good power to inhibit NF-kB and other pro-inflammatory cytokines (Bremner et al., 2009). The evaluation of *P. tridentatum* flower extracts' effects, concerning anti-inflammatory and anti-diabetic potential, should consider that luteolin was found to influence insulin action and production of adipokines/cytokines in adipocytes by activating the PPARγ pathway (Ding, Jin, & Chen, 2010), being important for type 2 diabetes and metabolic syndrome therapy. Luteolin was also found to possess anti-inflammatory activity (Ueda, Yamazaki, & Yamazaki, 2002). Accordingly, it is possible that several of the major constituents of our *P. tridentatum* flower extract (luteolin-*O*-(*O*-acetyl)-glucuronide and luteolin-*O*-glucuronide), despite being not identified by Vitor et al. (2004), can also be closely associated to decrease hyperglycemia in diabetic patients and to possess anti-inflammatory properties.

In Portuguese small villages, people often eat *P. tridentatum* yellow flowers which show resinous and astringent features. Hence, taking this into account and intending to evaluate also the toxicological effects of possible thermolabile compounds present in the yellow flowers, we used crude water extracts instead of decoctions.

Although this good correlation between *P. tridentatum* chemical composition and its large therapeutic use in traditional medicine, being one of the most required medicinal plants in Portuguese herb stores, no toxicological evaluation have been previously performed for this plant. In fact, for most aromatic and medicinal plants, evaluation of toxicological effects has started only in recent years (Aguilar-Santamaría et al., 2007; Déciga-Campos et al., 2007). Even so, for most plants its toxicological effects are still poorly described (Jordan et al., 2010; Street, Stirk, & Van Staden, 2008). Despite the secondary metabolites' toxicity (Sirikantaramas, Yamazaki, & Saito, 2008), other internal components can be toxic, as heavy metals often bio-accumulated by aromatic plants (Broadley et al., 2001; F.M. Ferreira et al., 2010). External factors, as molds, can also be a source of toxic compounds. Indeed, the presence of mycotoxins is a major problem for aromatic plants (Halt, 1998; Santos, Marín, Sanchis, & Ramos, 2009).

The assessment of *P. tridentatum* extracts' potential toxicological effects was performed evaluating several mitochondrial parameters (state 4 respiration, state 3 respiration, FCCP stimulated respiration, RCR and P/O ratio) from rat liver mitochondria. Consider that the liver is the major organ responsible for detoxification processes and mitochondria possess a central function in cellular energy production. In fact, mitochondrion is the major energy-producing organelle of the cell that also participates in multiple metabolic pathways. Thus, any interference with mitochondrial bioenergetics is known to be a part of cell injury process by a multiplicity of mechanisms and assorted agents (Krähenbühl, 2001; Wallace, 2008; Wallace et al., 1997). For that reason, novel high-throughput methods employ mitochondria as a bio marker for drug safety in pharmaceutical industry drug research (Dykens & Will, 2007). Furthermore, previous studies report the inhibitory effect of flavonoids on mitochondrial respiration (Dorta et al., 2005; Elingold et al., 2008). The respiratory chain inhibition promoted by several flavonoids has been frequently associated with the oxidation potentials of these compounds, which are commonly located in similar range as the mitochondrial redox centers (Firuzi, Lacanna, Petrucci, Marrosu, & Saso, 2005; Hodnick, Mllosavljevic, Nelson, & Pardini, 1988).

Our results showed that although RCR, P/O ratio and uncoupled respiration were not significantly affected by *P. tridentatum* extracts at the studied concentration range (0–500 mg.L^{-1}), state 4 and state 3 respiration rates decreased in some concentrations tested (from 50 to 500 mg.L^{-1}). This decline in respiratory activities is not consistent, and therefore, we hypothesized that instead of respiratory complexes' specific inhibition, this lower respiratory activity could be due to unspecific interactions of hydrophobic compounds with the mitochondrial inner membrane that could interfere with membrane fluidity (Kiebish, Han, Cheng, Chuang, & Seyfried, 2008). Indeed, swelling assay performed with K-acetate showed a slight increase in membrane permeability in the presence of *P. tridentatum* concentrations (250 or 500 mg.L^{-1}) commonly used in traditional medicine (or to flavor rice), however these declines are not reflected in phosphorylative efficiency.

These results indicate that *P. tridentatum* presents no toxicity *in vitro* for the tested conditions, since no specific interactions with respiratory enzymes were observed, and it neither diminished ΔpH in a significant way (as demonstrated through swelling assay). Furthermore, data of

cytotoxicity assay was consistent with the previous results, as the *P. tridentatum* extract (125 and 375 mg.L^{-1}) did not decrease the viability of HepG2 cells.

In conclusion, our results point out that *P. tridentatum* flowers can be suitably employed either in cooking or in traditional medicine, for treating ailments associated with oxidative stress, and this practice can be regarded as safe (at least for short-term therapies). Nonetheless, chronic toxicity cannot be excluded for long-term use, with particular relevance to the effects of the plant in intestinal mucosa (Sergent et al., 2008). Hence, careful assessment of *P. tridentatum* chronic toxicological potential is also required.

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